ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Third Quarterly Report of Progress

on

Research Project R-36-015-001

October 1 - December 31, 1965

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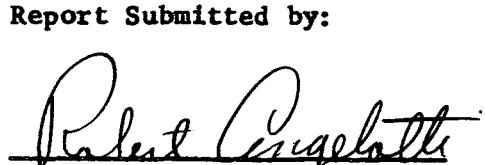

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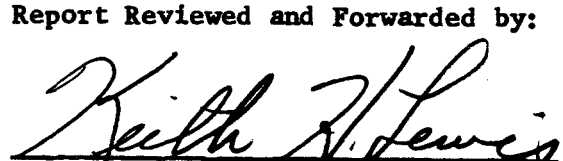

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SUMMARY

A method has been developed for formulating a clear, dense plastic rod (Lucite) free from bubbles which can be conveniently and consistently contaminated with Bacillus globigii spores and which appears suitable for studying the dry-heat resistance of these spores when they are encapsulated in the rods.

A method of recovering B. globigii spores from plastic rods has been developed and data have been collected indicating that the concentration of viable spores per gram of plastic does not change on prolonged storage at room temperature. This latter fact indicates that once polymerization is completed the plastic is not toxic for spores.

Statistical analysis of data relating to the reproducibility with which batches of plastic can be made up, contaminated, and formed into rods reveals that: (a) no difference in counts, on the average, occurs in rods prepared from separate batches of plastic on different days; (b) approximately the same number of spores, on the average, is present in every part of any rod when an inoculum level of at least 7×10^6 spores per gram is used.

Statistical analysis of data also revealed that the variation in counts between duplicate samples taken from a blender was significant and that an improved method for sampling the blend is necessary. Constant agitation of the blend during sampling would reduce this variation appreciably.

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INTRODUCTION

The results reported upon in the Second Quarterly Report of Progress indicated that it may be possible to introduce bacterial spores into certain types of plastics and recover the spores quantitatively. Efforts were expended this quarter in determining those factors that affect the persistence and quantitative recovery of spores incorporated into methyl methacrylate plastic (Lucite).

EXPERIMENTAL

Method of forming Lucite rods

Initial toxicity experiments conducted in a manner comparable to that described last quarter revealed that Lucite was non-toxic to Bacillus globigii spores. Because of the desirable chemical and physical properties of Lucite, as listed last quarter, it was selected as a model system for determining the heat resistance of B. globigii spore contamination encapsulated in it.

To remove the polymerization inhibitor, methyl methacrylate monomer is washed twice with equal volumes of 2% NaOH, followed by two additional washings with equal volumes of distilled water. The washed monomer is then mixed with an excess of anhydrous sodium sulfate (Na_2SO_4) and allowed to stand overnight to remove water. The sodium sulfate is removed by filtration and the monomer is stored in the cold (5°C) until ready for use.

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A series of experiments were conducted to determine the conditions necessary to obtain a clear plastic, free of bubbles, that would polymerize completely when exposed to 50°C for 2 hours. Higher temperatures and shorter times of polymerization were purposely avoided because of the possible effects such conditions might have on spores when introduced into plastic in future experiments.

The procedure finally adopted for forming Lucite rods contaminated with an acetone suspension of spores is as follows: 10 ml of the desired acetone suspension of spores is added to 50 ml of washed methyl methacrylate monomer and the two are mixed. To the mixture, 50 g of methyl methacrylate powder is added and mixed with a sterile spoon for 5 minutes. The inoculated unpolymerized (liquid) plastic is placed in a 250 ml Erlenmeyer vacuum flask and evacuated with a water pump until bubbles no longer form. The plastic, now viscous due to partial polymerization, is poured into cotton stoppered, sterile thermal-death-time borosilicate tubes which are placed in a 50°C water bath for 2 hours. Following polymerization, the thermal-death tubes, in which the solidified plastic has been molded, are sealed in an oxy-gas flame. This method consistently yielded plastic rods that were hard, clear, and free of bubbles.

Method for recovering B. globigii spores from plastic rods

The sealed thermal-death-time tubes are placed in a saturated iodine solution made up in 70% ethyl alcohol and exposed for 3 minutes. The tubes are removed aseptically and their surfaces dried with a sterile cloth towel. The towel is wrapped about the tube and the glass

is broken free from the molded plastic rod by tapping with a metal bar. One end of the free plastic rod is wrapped in a sterile paper towel and the rod is clamped in a vise. The rod is sawed with a hacksaw containing 10 sterile blades in alternating positions to achieve five separate blade cuts on each forward stroke and five separate blade cuts on each back stroke. Four cuts, approximately 1/2" wide and completely through the diameter, are made throughout the length of the rod in order to obtain representative sampling. The powdered plastic resulting from each cut is pooled and thoroughly mixed. One gram of the powder is placed in 200 ml of sterile acetone in a 400 ml Dessicoated beaker. The beaker is covered with sterile aluminum foil and placed on a magnetic mixer for 30 minutes at room temperature. During this interval, the plastic is completely dissolved. All of the 200 ml of acetone-plastic solution is passed through a sterile Dessicoated Seitz filter apparatus. The beaker is washed with an additional 200 ml of sterile acetone which is also passed through the filter and finally the filter funnel is rinsed with 100 ml of sterile acetone. The filter pad is placed in a Waring Blendor containing 500 ml of phosphate dilution water and ground for 2 minutes at slow speed, followed by one minute at high speed. Serial ten-fold dilutions of the paper pulp are plated, in duplicate, in typtone, glucose, beef extract agar. The plates are incubated 48 hours at 35°C and counted.

A number of experiments were performed to determine the percentage recovery of spores from plastic rods. Portions of aqueous stock spore suspensions were heat shocked at 80°C for 10 minutes and

diluted in sterile acetone so that 10 ml of acetone suspension contained the desired number of spores. Plastic rods were formed and analyzed as described above. The data presented in Table I are typical of the results obtained in several experiments. These data indicate that, as a rule, a reduction in the number of spores occurs immediately after addition to the plastic followed by an additional reduction during polymerization.

Distribution of spore contamination in plastic rods

Before quantitative survival data on spores encapsulated in plastic and subjected to dry heat can be collected and interpreted in a meaningful manner, the variables associated with the total system must be evaluated and their significance determined. For example, should uneven distribution of the spore contamination in a rod occur so that "pockets" of light and heavy contamination exist, heat treatment of such a sample would result in an apparent accomplished kill that would be considerably in error. To gather information on the factors that may contribute to variance, an experiment was devised by which a statistical determination of several variables was measured. The experimental design of this experiment is presented below.

Design for determining the distribution of spores in Lucite rods

Spore Conc.	Plastic Rod A_1				D A Y	Plastic Rod A_2				D A Y
	B_1	B_2	B_3	B_4		B_1	B_2	B_3	B_4	
C_1 D_1	a				3					5
D_2										
C_2 D_1					1					2
D_2										
C_3 D_1					4					6
D_2										

A_1 and A_2 = plastic rods, each from a different batch of plastic.

B_1, B_2, B_3, B_4 = four 1/2 inch sections sawed from different parts of the rod throughout its total length.

C_1, C_2, and C_3 = spore concentrations per gram of $1x10^6$, $1x10^4$, and $1x10^3$ respectively.

D_1 and D_2 = duplicate samples taken from the blender.

1 through 6 = days on which an experiment was performed.

a = duplicate plate count determinations.

For each of the three spore concentrations used, two separate batches of plastic were made on separate days and from these batches rods were formed and analyzed as described above. In order to randomize any day to day effects, the six experiments were performed in the random fashion shown below:

Day	Spore concentration per gram
1	1×10^4
2	1×10^4
3	1×10^6
4	1×10^3
5	1×10^6
6	1×10^3

The experiment was designed to examine the factors of: (A) duplicate rods mixed from different batches and on separate days; (B) parts of the rods averaged over all other effects; (C) concentration of spores per gram; (D) duplicate samples taken from the blender; and any of the paired interactions of the above four. An interaction will result when, for example, more spores are found in the top of a rod contaminated with a high concentration of spores and a large number of spores are also found in the bottom section of a rod contaminated with a low concentration of spores. This design is a $2^3 x 4$ factorial with two duplicate plates at each combination of the four factors. Ostle (1963) gives a general description of the analysis, and states the assumptions for this type of design.

Plate counts from the appropriate dilutions of the two concentrations were transformed to logarithms to the base 10 in order to yield approximate normality.

As a further test of the assumptions listed by Ostle, a Bartlett's test for homogeniety of individual duplicates at each combination of factors was performed and found to be non-significant at the $\alpha = 0.05$ level. The significance level of all tests was chosen as $\alpha = 0.05$, where α is the probability of rejecting a true hypothesis.

Because of the small numbers of spores present per ml of blend derived from the rods with the lowest spore concentration, dilutions were not made, but instead, three 4-ml portions of the blend were plated. Counts obtained from these 3-ml portions varied too widely to be analyzed with data from the two other experiments. Subsequent analysis of the data from the rods inoculated with the two higher

concentration of spores (see below) indicated that a large portion of the variance was due to differences between counts on samples taken from the blender (factor D).

Table II lists the numbers of spores used to inoculate the plastic rods and the numbers of spores recovered before and after polymerization of the plastic. The results from the analysis of variance are shown in Table III as well as all of the factors and the paired combinations or interactions and the variation (mean square) of each. The error mean square was obtained by pooling the sum of squares due to duplicates, with higher order interactions, and dividing by 45 degrees of freedom. All of the other factors and interactions were compared to the error by dividing the error mean square into each of the other mean squares to obtain the F-ratios shown in the last column. This value of the F-ratio was compared to values in tables for the proper number of degrees of freedom (Ostle, 1963). Five of the factors and interactions were found to be significant at $\alpha = 0.05$.

The significant tests indicated the following:

1. The counts obtained from different parts of the rod (factor B) are significant, on the average, over the other factors. A test of three individual degrees of freedom showed that the top two sections of the rod were higher in count on the average than the bottom two sections.
2. The plate counts (factor C) obtained from the dilutions of the rods with the highest spore concentration were higher than those for the lower concentrations.

3. The second sample (factor D) taken from the blender has a higher average count than the first sample.

4. The "concentrations" x "part of rod" interaction (BC) is significant. A graph of the means of all the levels of the two factors indicates that those rods with the highest spore concentration showed no difference in spore distribution from top to bottom while the rods of lower concentration yielded lower counts at the bottom of the rod.

5. The "part of rod" x "samples from blender" interaction (BD) showed the same trend as in 4 above. The second sample taken from the blender showed little difference in counts from the top and bottom parts of the rod, whereas the first sample, having a lower concentration, showed a difference in the average counts between the top and bottom of the rod.

Persistence of plastic encapsulated spores held at room temperature

Previous contractors to NASA (Koesterer, 1965) have reported rather high D_{125} values for spores encapsulated in two types of plastic material. From these data, one is able to estimate that approximately 20 to 30 hours exposure to a dry heat temperature of 125°C would be required to reduce $1x10^8$ Bacillus globigii spores to non-detectable levels. Because of such extended exposure times, it is necessary to know whether any death of spores occurs in the plastic on extended storage as a result of plastic toxicity. The extent of non-thermal death as well as any changes in the rate of such death would have to be understood and taken into account when calculating thermal resistance of spores encapsulated

in plastic. To determine whether any change in the number of viable spores encapsulated in plastic would occur over an extended storage period, the following experiment was performed. Rods of plastic containing approximately 220×10^6 spores per gram were formed as described above. Immediately after polymerization, a rod was sampled by the method described above and the number of spores per gram determined. The remaining rods were stored at room temperature, and at various intervals, individual rods were similarly sampled and the number of spores per gram determined. The results of this experiment are given in Table IV which reveals that the number of spores per gram remains fairly uniform over a 72-hour period.

PROJECTED RESEARCH FOR FOURTH QUARTER

Activities during the fourth quarter will be largely devoted to obtaining thermal resistance data on B. globigii spores incorporated in Lucite at a concentration of 1×10^8 spores per gram and at a dry heat temperature of 125°C.

REFERENCES

1. Ostle, Bernard, "Statistics in Research", 2nd Edition, Iowa State University Press, Ames, Iowa, 1963.

2. Koesterer, M. G., NASA Contractor Report CR-191, March 1965.

Table I

Recovery of Bacillus globigii spores from Lucite

Experiment number	No. of spores per gram of plastic*	No. of spores per gram recovered from unpolymerized plastic	No. of spores per gram recovered from polymerized plastic	Percent recovered ($\frac{\text{Polymerized}}{\text{Unpolymerized}}$)
I	350×10^6	163×10^6	120×10^6	73
II	5×10^6	5×10^6	5×10^6	100
III	3.6×10^8	3.3×10^8	2.6×10^8	78
IV	6.8×10^5	6.3×10^5	2.5×10^5	40

*Calculated from the plate count value obtained on the acetone suspension used to inoculate the plastic.

Table II

Distribution of Bacillus globigii spore contamination in Lucite rods prepared from several batches of plastic.

Day of the Experiment	No. of spores added per gram of plastic[a]	No. of spores per gram recovered from unpolymerized plastic[b]	No. of spores recovered from polymerized plastic[c]	Average percent recovered (Polymerized / Unpolymerized)
6	9.1×10^3	1.3×10^3 1.0×10^3 Av. 1.5×10^3	336 840 672 924 Av. 696	61
4	9.5×10^3	1.0×10^3 588 Av. 794	504 84 924 420 Av. 483	61
1	35.5×10^4	41×10^4 38×10^4 Av. 39.5×10^4	20×10^4 23×10^4 28×10^4 28×10^4 Av. 24.8×10^4	62
3	5.5×10^6	8.3×10^6 6.8×10^6 Av. 7.6×10^6	5.9×10^6 5.5×10^6 6.4×10^6 4.9×10^6 Av. 5.7×10^6	75
5	6.4×10^6	7.9×10^6 7.4×10^6 Av. 7.7×10^6	5.9×10^6 4.3×10^6 5.9×10^6 5.7×10^6 Av. 5.5×10^6	71
2	20.2×10^4	18.8×10^4 31.9×10^4 Av. 25.4×10^4	25.5×10^4 21.7×10^4 23.7×10^4 25.8×10^4 Av. 24.2×10^4	95

[a] Calculated from the plate count value obtained on the acetone suspension of spores used to inoculate the plastic.

[b] Values obtained from two portions of unpolymerized plastic immediately after inoculation and mixing.

[c] Values obtained from plastic cut from four different sections along the rod length.

Table III

Analysis of Variance of Data From an Experiment With Spores in Plastic Rods

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Ratio
Duplicate Rods A	0.00281	1	0.00281	0.39
Parts of Rod B	0.07426	3	0.02475	3.45*
Concentration C	1.73778	1	1.73778	242.03*
Duplicates From Blender D	0.03780	1	0.03780	5.26*
Interactions AB	0.00260	3	0.00086	0.12
AC	0.02685	1	0.02685	3.74
AD	0.00614	1	0.00614	0.86
BC	0.07502	3	0.02500	3.48*
BD	0.07849	3	0.02616	3.64*
CD	0.00454	1	0.00454	0.63
Error	0.32328	45	0.00718	----
TOTAL	2.36957	63	----	----

*Significant at $\alpha = 0.05$

Table IV

Effect of Room Temperature Storage on the Viability of *Bacillus globigii* Spores Incorporated in Lucite Rods

Hours of Storage at Room Temperature	No. of Spores Detected Per Gram of Plastic ($X10^6$)
0*	120
12	122
24	124
48	90
72	126

*The 0-hour determination was made immediately after polymerization was complete. Complete polymerization occurs in 2 hours in a 50°C water bath.